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Research Article

Two novel *Tuber* species (Tuberaceae, Pezizales) from southwestern China based on morphological and molecular evidence

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Abstract

Based on morphological and molecular phylogenetic analyses, two new species, *Tuber marroninum* and *Tuber conditum*, belonging to the Latisporum group, were described from Yunnan province of southwest China. Morphologically, *Tuber marroninum* is distinguished from other Latisporum species by whitish and glabrous ascomata and a two-layered peridium, measuring 150–200 µm in total thickness, with an outer layer ranging from 40–60 µm. *Tuber conditum* is diagnosed by its thinner peridium (120–200 µm) and larger ascospores (49–66.5 × 34.5–60 µm in one-spored asci). According to the outcome of the ITS rDNA sequence analysis, the species of *T. marroninum* and *T. conditum* each form a distinct and well-supported group within the Latisporum group, respectively.

Key words: Ascomycetes, Latisporum group, new taxa, phylogenetic analysis, taxonomy

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Introduction

Tuber F.H. Wigg (Tuberaceae) is one of the most important genera in Pezizales in terms of both ecology and economy. It is widespread in Asia, Europe, and North America (Mello et al. 2006; Jeandroz et al. 2008; Trappe et al. 2009; García-Montero et al. 2010; Bonito et al. 2013; Zambonelli et al. 2016), with a few species also occurring in North Africa (Cacialli 2005). *Tuber* spp. establish obligate mycorrhizal symbiotic relationships with diverse plant hosts, such as oaks, pines, poplars, and commercially important trees, including chestnut, pecan, and hazelnut (Geng et al. 2009; Trappe et al. 2009; Wan et al. 2015). Not only can mycelium of *Tuber* spp. provide water and mineral nutrition for their host trees, but these fruiting bodies also contribute to the diet of small forest mammals. Some mycophagous animals are considered major consumers and vectors of hypogeous ectomycorrhizal fungi (Schickmann et al. 2012). Additionally, the fruiting bodies of some species, such as *Tuber magnatum*, *T. melanosporum*, *T. aestivum*, and *T. indicum*, have high value as a delicious food because of their unique scent. Although edible fungi are an important component of Chinese culture, there appear never to be accounts of *Tuber* species in ancient Chinese literature (Wang and Liu 2009). *Tuber taiyuanense* was the first reported species in 1985 in China (Liu 1985). Results of the last 20 years of

research have revealed that China is a rich country in truffle diversity (Wang and Liu 2009). More than 60 truffle species have been documented in China, with the majority classified into the Puberulum, Latisporum, Maculatum, Rufum, *and* Melanosporum groups (http://www.speciesfungorum.org/Names/Names.asp). *Tuber* species in the Puberulum, Maculatum, and Gibbosum groups were commonly called "whitish truffle" to distinguish them from Italian white truffle, *T. magnatum* (Bonito et al. 2010; Lancellotti et al. 2016). The Rufum and Melanosporum groups are called "black truffle" (Bonito et al. 2010). In this study, two whitish new truffle species have been discovered in Yunnan province, China.

Materials and methods

Morphological studies

Fresh samples were collected from forests of *Pinus yunnanensis* Franch. in Yunnan, China. Microscopic and macroscopic characteristics are described based on the specimen materials (L3694 and L3385) following the methods of Yang and Zhang (2003). Color codes were given based on Kornerup and Wanscher (1978). Sections were made with a razor blade by hand, mounted in a 5% (w/v) KOH solution, then lactophenol cotton blue, and examined under an OLYMPUS BH-2 microscope. For scanning electron microscopy (SEM), ascospores were scraped from the dried gleba onto double-sided tape and mounted directly on an SEM stub. They were then coated with gold-palladium, examined, and photographed with a JEOL, JMS-5600LV SEM. For evaluation of the range of ascospore size, at least 40 ascospores each from one specimen of each collection site were measured. For ascospores, *Q*, the length-to-width ratio, is given in the same format as spore dimensions, and ***Q*** is the average *Q* of all specimens ± standard deviation. The specimens were deposited in the herbarium of the Biotechnology and Germplasm Resources Institute, Yunnan Academy of Agricultural Sciences (YAAS), and the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (HKAS).

DNA extraction, PCR, and sequencing

Total DNA was extracted from pieces of dried ascomata with a modified CTAB methodology (Hofstetter et al. 2002, Li et al. 2011). Polymerase chain reactions (PCR) were performed using the primer combination ITS4/ITS5 (Hofstetter et al. 2002, Larsson and Sundberg 2011), nuc 28S rDNA subunit (28S) were amplified with primers LROR/LR5 (Vilgalys and Hester 1990), bRPB2-5F/bRPB2-7.1R (Matheny 2005) were used to amplify the second-largest subunits of RNA polymerase II (RPB2) (Sung et al. 2007) and 983F/2212R (Rehner and Buckley 2005) were amplified with primers translation elongation factor 1-α gene (tef-1α). In 25 µL of PCR reaction solution were contained 1 µL of DNA, 1 µL (5 µm) of each primer pair, 2.5 µL of 10 × buffer (Mg^{2+} plus), 1 µL of dNTP (1 mM), 0.5 µL of BSA (0.1%), 0.5 µL of $MgCl_2$, and 1 U of Taq DNA polymerase (Takara Tag, Takara Biotechnology, Dalian, China). PCR reactions were run as follows: for the ITS gene, 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 1 min, and 72 °C for 1 min. The final reaction was followed by an extension at 72 °C for 10 min. For the LSU gene, 94 °C for 5 min, followed by 35 cycles of

94 °C for 1 min, 60 °C for 50 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were sent to Sangon Biotech Corporation (Shanghai, China) for purifying and sequencing by using ITS4/ITS5, LROR/LR5, bRPB2-5F/bRPB2-7.1R, and 983F/2212R, respectively.

Phylogenetic analyses

The *Tuber* sequences for phylogenetic analysis were obtained in this study and from the GenBank database. A total of 210 sequences (including 202 sequences by collections of *Tuber* and 8 sequences by sequencing in this study) (Table 1), in which 5 sequences derived from two species of *Choiromyces helanshanensis* and *C. meandriformis* were selected and used as outgroups (Figs 1, 2). Sequences were edited and assembled using SeqMan Pro (DNAStar, Inc.). Alignment was performed using the online version of the multiple sequence alignment program MAFFT v7 (Katoh and Standley 2013), applying the G-INS-I strategy, and the alignments were manually adjusted in BioEdit. Poorly aligned sites were identified, and ambiguous sites were excluded by Gblocks v. 0.91b (Castresana 2000; using default options except "Allowed Gap Postions" = half) with default parameters. The multiple sequence alignments of both ITS-LSU-RPB2-TEF1 and ITS1 + 5.8S + ITS2 (based on the sequence of *Tuber glabrum* NR_153217, Fan et al. 2014) were carried out, establishing the phylogenetic trees, respectively. A maximum likehood (ML) phylogenetic tree was constructed using RAxML v7.0.3 on the online server accessed at https://mafft.cbrc.jp/alignment/server/, applying the rapid bootstrapping algorithm for 1000 replications using the GTRGAMMA model (Stamatakis et al. 2005; Stamatakis 2014). Clades with bootstrap values (BS) ≥ 75% were considered as significantly supported (Hillis and Bull 1993). Bayesian inference (BI) was performed using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method in MrBayes version 3.2.7a (Ronquist et al. 2012), and the GTR + I + G model was selected as the best model under the Akaike Information Criterion (AIC) implemented by MrModeltest v.2.3 (Nylander 2004). Two independent runs of chains were conducted for 2,000,000 (ITS-nrLSU-RPB2-TEF1) and 1,000,000 (ITS1 + 5.8S + ITS2). The average standard deviations of split frequencies (ASDSF) were less than 0.01 at the end of the run, and ESS (effective sampling size) values were more than 200. Trees were sampled every 100 generations after burn-in (well after convergence), and 50% majority-rule consensus trees were constructed and viewed with TreeView32 (Page 2001). Bayesian posterior probabilities (PP) ≥ 0.90 were considered as significant support (Alfaro et al. 2003).

Results

Molecular phylogenetics

The phylograms of ITS-nrLUS-RPB2-TEF1 (446 bp, 850 bp, 902 bp, and 814 bp, respectively) and ITS1 + 5.8S + ITS2 (446 bp) sequences are shown in Fig. 1, and Fig. 2, respectively. ML and BI analyses produced similar tree topologies, and only the tree generated from the ML analysis is shown. Both phylograms indicated that specimens of *T. marroninum* and *T. conditum* form a monophyletic clade, which was distinct from other *Tuber* species with high BS and PP support.

Table 1. Details of the *Tuber* sequences used in phylogenetic analysis. Sequences newly generated for this study are in bold.

Name	Voucher/isolate/strain	ITS	LSU	RPB2	TEF1
C. helanshanensis	HKAS_80633	KU531604	OM366182	OM584242	OM649586
C. helanshanensis	HKAS_80630	KU531603	OM366202	OM584265	OM649607
C. helanshanensis	HKAS_80634	NR_153899	OM366156	OM584208	OM649562
Choiromyces meandriformis	RH691	HM485330	FJ809794	JQ954471	JX022550
C. meandriformis	GB285	HM485331	OM366158	OM584209	OM649563
T. alboumbilicum	YAAS_L2324	NR_155904	OM366174	OM584231	OM649578
T. anniae	JT13209	HM485338	JQ925680	/	JX022567
T. anniae	BJTC_FAN640	OM286868	OM366215	OM584274	OM649620
T. baoshanense	BJTC_FAN400	OM256791	OM366197	OM584260	OM649602
T. baoshanense	HKAS_88788	NR_178114	OM366176	OM584233	OM649580
T. borchii	IFS Y. Wang 89556	DQ478624	OM366222	OM584280	OM649629
T. borchii	IFS Y. Wang 89377	DQ478656	/	/	/
T. badium	BJTC FAN381	OM256748	OM366193	OM584256	OM649598
T. badium	BJTC FAN371	OM256747	OM366192	OM584255	OM649597
T. borchii	BJTC FAN217	KT067681	KT067706	OM584229	KT067717
T. borchii	Tbo1	MZ452886	/	MZ541897	MZ570608
T. brumale	GB53	FJ748900	/	/	/
T. brumale	Tbru-eu02	DQ329359	/	/	/
T. brumale	T.bru-eu03	DQ329360	/	/	/
T. brunneum	JT33836	KT897475	/	/	/
T. brunneum	JT33837	KT897479	/	/	/
T. buendiae	MUB_Fung-0974	NR_171295	/	/	/
T. buendiae	MUB_Fung-0976	MT006097	/	/	/
T. buendiae	MUB_Fung-0977	MT006098	/	/	/
T. californicum	JT28058	HM485346	JQ925685	JQ954496	JX022574
T. californicum	JT22590	HM485351	/	/	/
T. calosporum	HKAS_88791	KT444597	/	/	/
T. calosporum	HKAS_88790	KT444598	/	/	/
T. canaliculatum	OSC59072	HM485347	/	JQ954498	JX022576
T. canaliculatum	JT28215	JQ925643	/	JQ954497	JX022575
T. candidum	JT21856	HM485348	/	/	/
T. candidum	JT1844	HM485349	/	/	/
T. caoi	BJTC_FAN293	KP276182	KP276198	OM584240	KP276217
T. caoi	BJTC_FAN309	KP276181	/	/	/
T. caoi	BJTC_FAN321	KP276180	/	/	/
T. caoi	BJTC_FAN271	KP276183	NG_059984	OM584237	KP276216
T. crassitunicatum	BJTC_FAN465	MH115295	NG_088329	OM584268	OM649610
T. crassitunicatum	HMAS_88577	MH115297	/	/	/
T. dryophilum	GB35	JQ925644	JQ925687	/	JX022577
T. dryophilum	GB37	HM485354	JQ925688	JQ954501	JX022578
T. ferrugineum	JST22014	KX354297	/	/	/
T. ferrugineum	MUBFung-0972	MN962719	/	/	/
T. foetidum	ZB2452	JQ288905	/	/	/
T. foetidum	ZB2489	JQ288906	/	/	/
T. formosanum	BJTC_FAN107	MF621549	OM366159	OM584210	OM649564
T. formosanum	BJTC_FAN356	MF627986	OM366189	OM584252	OM649594
T. furfuraceum	HKAS_48272	GU979034	/	/	/
T. furfuraceum	HKAS_49707	GU979049	/	/	/
T. glabrum	BJTC_Fan228	NR_153217	NG_088326	OM584234	OM649581
T. glabrum	BJTC_Fan232	KF002727	OM366179	OM584236	/
T. griseolivaceum	BJTC_FAN469	KY428921	NG_088330	/	OM649612
T. griseolivaceum	BJTC_FAN470	KY428923	/	/	/

Name	Voucher/isolate/strain	ITS	LSU	RPB2	TEF1
T. himalayense	CB20001	MW393547	/	/	/
T. himalayense	JJ22045	OQ054615	/	/	/
T. himalayense	JJ22046	OQ054619	/	/	/
T. hubeiense	HMAS_60233	KT067688	/	/	/
T. huidongense	BJTC_FAN103	MH115294	/	/	/
T. huidongense	885-1	MW201514	/	/	/
T. huizeanum	BJTC_FAN314	KT067685	KT067692	OM584246	KT067712
T. huizeanum	BJTC_FAN186	JQ910651	NG_059991	/	OM649575
T. indicum	T47	JQ639005	/	/	/
T. indicum	T49	JQ639007	/	/	/
T. jinshajiangense	BJTC_FAN124	KP276177	/	/	/
T. jinshajiangense	BJTC_FAN406	KX575841	OM366199	OM584262	OM649604
T. jinshajiangense	BJTC_FAN407	KX575842	OM366200	OM584263	OM649605
T. latisporum	BJTC_FAN126	KP276189	KP276204	OM584215	KP276205
T. latisporum	BJTC_FAN125	KT067676	KT067695	OM584214	KT067725
T. liaotongense	OSC87602	HM485369	FJ809813	/	JX022589
T. liaotongense	BJTC_FAN550	MH115302	OM366213	OM584272	OM649618
T. lijiangense	BJTC_FAN307	KP276188	KP276203	OM584244	KP276206
T. lishanense	BJTC_FAN683	MH115305	MH115306	OM584275	OM649621
T. lishanense	BJTC_FAN718	NR_160619	NG_064527	OM584276	OM649622
T. liui	HXZE_984	DQ478636	/	/	/
T. liui	HKAS_48269	DQ898182	/	/	/
T. liyuanum	FAN162	JQ771191.2	KT067698	OM584218	KT067710
T. liyuanum	BJTC_FAN187	JQ771193	KT067704	/	KT067719
T. longispinosum	FFPRI_460530	LC508589	/	/	/
T. longispinosum	FFPRI_460528	LC508587	/	/	/
T. longispinosum	FFPRI_460529	LC508588	/	/	/
T. lyonii	FLASMES-614	MT156453	/	/	/
T. lyonii	FLASMES-615	MT156454	/	/	/
T. macrosporum	JT13362	HM485373	FJ809838	/	JX022590
T. macrosporum	JT19458	HM485372	/	/	/
T. maculatum	BJTC_FAN868	OM265274	OM366227	OM584283	OM649634
T. maculatum	BJTC_FAN876	OM265278	OM366228	OM584284	OM649635
T. malacodermum	JT32319	FJ809889	JQ925702	JQ954514	JX022593
T. marroninum	**YAAS_L3694**	**ON454668**	**ON428904**	**OQ305202**	**OQ305199**
T. marroninum	**YAAS_L3695**	**OQ297680**	/	/	/
T. melanosporum	MEL437_clone_25A	EU555389	/	/	/
T. melanosporum	MEL437_clone_26A	EU555390	/	/	/
T. microsphaerosporum	BJTC_Fan152	KF805726	/	/	/
T. microsphaerosporum	BJTC_FAN152	KP276187	/	/	KP276207
T. microspiculatum	BJTC_FAN220	MH115315	/	/	/
T. microspiculatum	BJTC_FAN138	MH115317	/	/	/
T. nitidum	BM105	FJ809885	FJ809807	JQ954517	JX022597
T. nitidum	MUB:Fung-0934	MN962721	/	/	/
T. nitidum	MUB:Fung-0935	MN962722	/	/	/
T. oligospermum	AH39336	JN392267	/	/	/
T. oligospermum	AH39337	JN392268	/	/	/
T. oligospermum	AH39338	JN392266	/	/	/
T. panzhihuanense	DXJ277	JQ978651	/	/	/
T. panzhihuanense	DXJ278	JQ978652	/	/	/
T. panzhihuanense	HKAS_72015	NR_120126	/	/	/
T. parvomurphium	BJTC_FAN323	KP276185	KP276191	/	KP276215
T. parvomurphium	BJTC_FAN298	KP276186	NG_059981	OM584241	KP276214
T. piceatum	HMAS_97125	NR_160620	/	/	/

Name	Voucher/isolate/strain	ITS	LSU	RPB2	TEF1
T. piceatum	HMAS_97124	MH115320	/	/	/
T. polymorphosporum	HKAS_88793	NR_178113	/	/	/
T. polymorphosporum	BJTC_FAN413	OM256794	/	/	/
T. polymorphosporum	BJTC_FAN544	OM256802	/	/	/
T. pseudobrumale	BJTC_FAN306	OM287838	/	/	/
T. pseudobrumale	BJTC_FAN322	OM287839	/	/	/
T. pseudobrumale	YAAS_L3181	KJ742703	/	/	/
T. pseudoexcavatum	CJ408	HM485381	/	/	/
T. pseudoexcavatum	T14_HKAS_44325b	GU979039	/	/	/
T. pseudoexcavatum	T29_HKAS_44324b	GU979040	/	/	/
T. pseudoexcavatum	HKAS_44346	GU979041	/	/	/
T. pseudoexcavatum	T18_HKAS_49851	GU979043	/	/	/
T. pseudoexcavatum	T31_HKAS_47617	GU979045	/	/	/
T. pseudoexcavatum	T80_HKAS_49747	GU979046	/	/	/
T. pseudoexcavatum	HKAS 86489	MG871289	/	/	/
T. pseudohimalayense	BJTC_FAN122	MF627983	/	/	/
T. pseudohimalayense	BJTC_FAN297	OM287837	/	/	/
T. pseudohimalayense	BJTC_FAN328	OM287840	/	/	/
T. pseudohimalayense	BJTC_FAN354	OM287844	/	/	/
T. pseudohimalayense	BJTC_FAN541	OM287847	/	/	/
T. pseudohimalayense	mhll-10	MT446223	/	/	/
T. pseudohimalayense	mhll-11	MT446224	/	/	/
T. pseudomagnatum	BJTC_FAN163	JQ771192	KP276192	OM584219	KP276208
T. pseudomagnatum	BJTC_FAN299	KP276184	/	/	/
T. pseudomagnatum	BJTC_FAN391	OM265244	OM366195	OM584258	OM649600
T. pseudosphaerosporum	BJTC_FAN250	NR_153229	NG_059982	/	OM649584
T. pseudosphaerosporum	BJTC_FAN394	OM256789	/	/	/
T. puberulum	ZB1341	JF261381	/	/	/
T. puberulum	ZB1433	JF261382	/	/	/
T. pustulatum	AQUI_9725	MK211278	/	/	/
T. pustulatum	AQUI_9726	MK211279	/	/	/
T. rapaeodorum	IFS Y. Wang 89719	DQ478651	/	/	/
T. rapaeodorum	IFS Y. Wang 870202	DQ478654	/	/	/
T. qujingense	HKAS 95823	KX904885	/	/	/
T. rapaeodorum	ZB452	JF261396	/	/	/
T. rapaeodorum	ZB466	JF261397	/	/	/
T. regimontanum	ITCV_909	EU375838	NG_059920	JQ954520	JX022600
T. rufum	FLAS-F-65583	MT374049	/	/	/
T. rufum	FLAS-F-65584	MT374050	/	/	/
T. shidianense	HKAS_88770	KT444595	/	/	/
T. shidianense	HKAS_88771	KT444596	/	/	/
T. sinense	BJTC_FAN108	MF627968	OM366160	OM584211	OM649565
T. sinense	BJTC_FAN110	MF627970	OM366161	OM584212	OM649566
T. sinoalbidum	BJTC_FAN105	MH115298	/	/	/
T. sinomonosporum	BJTC_Fan150	KF002729	/	/	/
T. sinosphaerosporum	BJTC_FAN136	JX092087	KP276196	/	KP276211
T. sinosphaerosporum	BJTC_FAN135	JX092086	NG_059983	OM584217	OM649569
Tuber sp. 2 *KA-2010*	K22	AB553345	/	/	/
Tuber sp. 2 *KA-2010*	K108	AB553349	/	/	/
Tuber sp. 2 *KA-2010*	K205	AB553353	/	/	/
Tuber sp. 2 *KA-2010*	K445	AB553366	/	/	/
Tuber sp. 2 *KA-2010*	K446	AB553367	/	/	/
T. songlu	HKAS_95771	KX904883	/	/	/
T. songlu	HKAS_95777	KX904884	/	/	/

Name	Voucher/isolate/strain	ITS	LSU	RPB2	TEF1
T. songlu	HKAS_95851	KX904886	/	/	/
T. sphaerosporum	JT12487	FJ809853	FJ809805	/	JX022609
T. sphaerosporum	JT19772	FJ809854	/	/	/
T. conditum	**YAAS_L3385**	**ON454665**	**ON428901**	**/**	**/**
T. conditum	**YAAS_L3682**	**ON454667**	/	/	/
T. spinoreticulatum	RH158	GQ221454	/	/	/
T. spinoreticulatum	U188	FJ809884	FJ809815	JQ954527	JX022608
T. subglobosum	BJTC_FAN222	KF002728	OM366175	OM584232	OM649579
T. subglobosum	BJTC_FAN432	MH115323	/	/	/
T. taiyuanense	T42_HM75888	GU979033	/	/	/
T. taiyuanense	BJTC_FAN210	OM311199	/	/	/
T. texense	JT15162	HM485391	/	/	/
T. thailandicum	CMU-B31	MW470964	/	/	/
T. thailandicum	CMU-B33	MW470967	/	/	/
T. theleascum	AQUI_9729	MK211283	/	/	/
T. theleascum	AQUI_9730	MK211284	/	/	/
T. theleascum	ITCV_908	NR_164592	/	/	/
T. tomentosum	K126	AB553447	/	/	/
T. tomentosum	K200	AB553449	/	/	/
T. tomentosum	K409	AB553450	/	/	/
T. tomentosum	K410	AB553451	/	/	/
T. tomentosum	K412	AB553452	/	/	/
T. tomentosum	K413	AB553453	/	/	/
T. tomentosum	K414	AB553454	/	/	/
T. tomentosum	K415	AB553455	/	/	/
T. tomentosum	K199	AB553448	AB553521	AB553561	AB553541
T. pseudoexcavatum	**YAAS_L3932**	**ON454670**	**/**	**/**	**OQ305200**
T. pseudoexcavatum	**YAAS_L3933**	**OQ297681**	/	/	/
T. umbilicatum	T2_HKAS_44316	GU979031	/	/	/
T. umbilicatum	T30_HKAS_48267	GU979032	/	/	/
T. umbilicatum	BJTC_FAN225	MH115325	/	/	/
T. variabilisporum	BJTC_FAN330	OM287841	/	/	/
T. vesicoperidium	BJTC_FAN155	JQ690071	JQ690068	/	KP276212
T. vesicoperidium	L156	JQ690072	/	/	/
T. wanglangense	IFS Y.Wang_610	DQ478637	/	/	/
T. wenchuanense	HMAS_60239	JX267044	/	/	/
T. wenchuanense	BJTC_FAN833	OM311256	/	/	/
T. calosporum	**YAAS_L3648**	**ON454666**	**ON428902**	**OQ305201**	**OQ305198**
T. calosporum	**YAAS_L3684**	**OQ297679**	/	/	/
T. xuanhuaense	BJTC_FAN618	MK045627	OM366214	OM584273	OM649619
T. xuanhuaense	BJTC_FAN887	MK045644	/	/	/
T. xuanhuaense	BJTC_FAN886	MK045645	/	/	/
T. xuanhuaense	HMAS_60213	NR_147436	/	/	/
T. yigongense	BJTC_FAN731	MF663714	/	/	/
T. yigongense	BJTC_FAN729	MF663716	/	OM584277	OM649623
T. zambonelliae	MUB_Fung-1006	MW632954	/	/	/
T. zambonelliae	MUB_Fung-1007	MW632955	/	/	/
T. zambonelliae	MUB_Fung-0995	NR_174649	/	/	/
T. zhongdianense	BJTC_FAN176	KP276178	/	/	/
T. zhongdianense	BJTC_FAN178	KT067679	/	/	/
T. zhongdianense	BJTC_FAN182	KT067680	KT067702	/	KT067721
T. zhongdianense	BJTC_FAN189	KT067689	KT067705	/	KT067718

Note: The symbol "/" representative was not detected, and the black fonts showed the object of study in this paper.

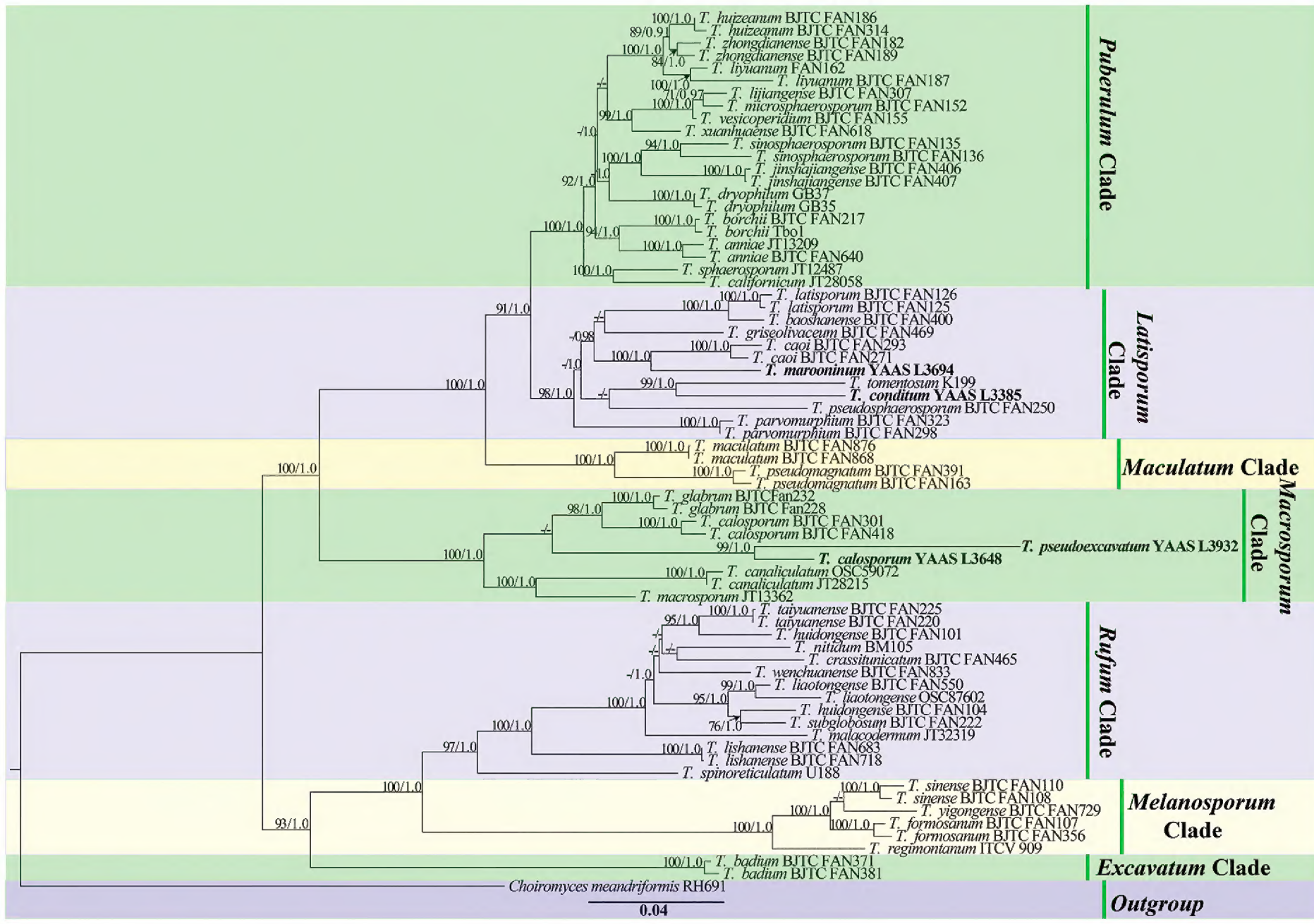

Figure 1. Phylogeny generated from maximum likelihood and Bayesian inference analysis of the ITS-LUS-TEF1-RPB2 rDNA sequences from *Tuber marroninum* and *T. conditum*-related species. *Choiromyces helanshanensis* and *C. meandriformis* served as outgroups. ML bootstrap values (>75%) and Posterior Probabilities (PPs) values (>0.90) are shown above or beneath the branches at nodes. Novel sequences are printed in bold.

Based on the ITS1 + 5.8S + ITS2 analysis (Fig. 2), *T. marroninum* and *T. conditum* stand with the Latisporum clade together with *Tuber tomentosum*, *T. rapaeodorum*, *T. songlu*, *T. thailandicum*, *T. pseudosphaerosporum*, *T. polymorphosporum*, *T. panzhihuanense*, *T. lastisporum*, *T. baoshanense*, *T. parvomurphium*, *T. alboumbilicum*, *T. griseolivaceum*, and *T. caoi*, and the phylogenetic tree showed their strong bootstrap support values (BS = 100% and PP = 1.0). In this study, two additional specimens of *Tuber pseudoexcavatum* and *Tuber calosporum* were collected through morphological and molecular identification (BS = 100% and PP = 1.0) and preserved in the Herbarium of the Institute of Forestry and Soil Sciences (YAAS), implying these two species might be common in the southwest region of China.

Taxonomy

***Tuber marroninum* T. J. Yuan, S. H. Li & X. H. Wang, sp. nov.**

MycoBank No: 901881

Fig. 3a–g

Typification. China • Yunnan Province: Xiangyun County (25°20'768"N, 100°43'34"E), in soil under mixed forest, with *Pinus yunnanensis* dominant, 20 Sep 2020, S. H. Li L3694 (holotype YAAS L3694, paratype YAAS L3695).

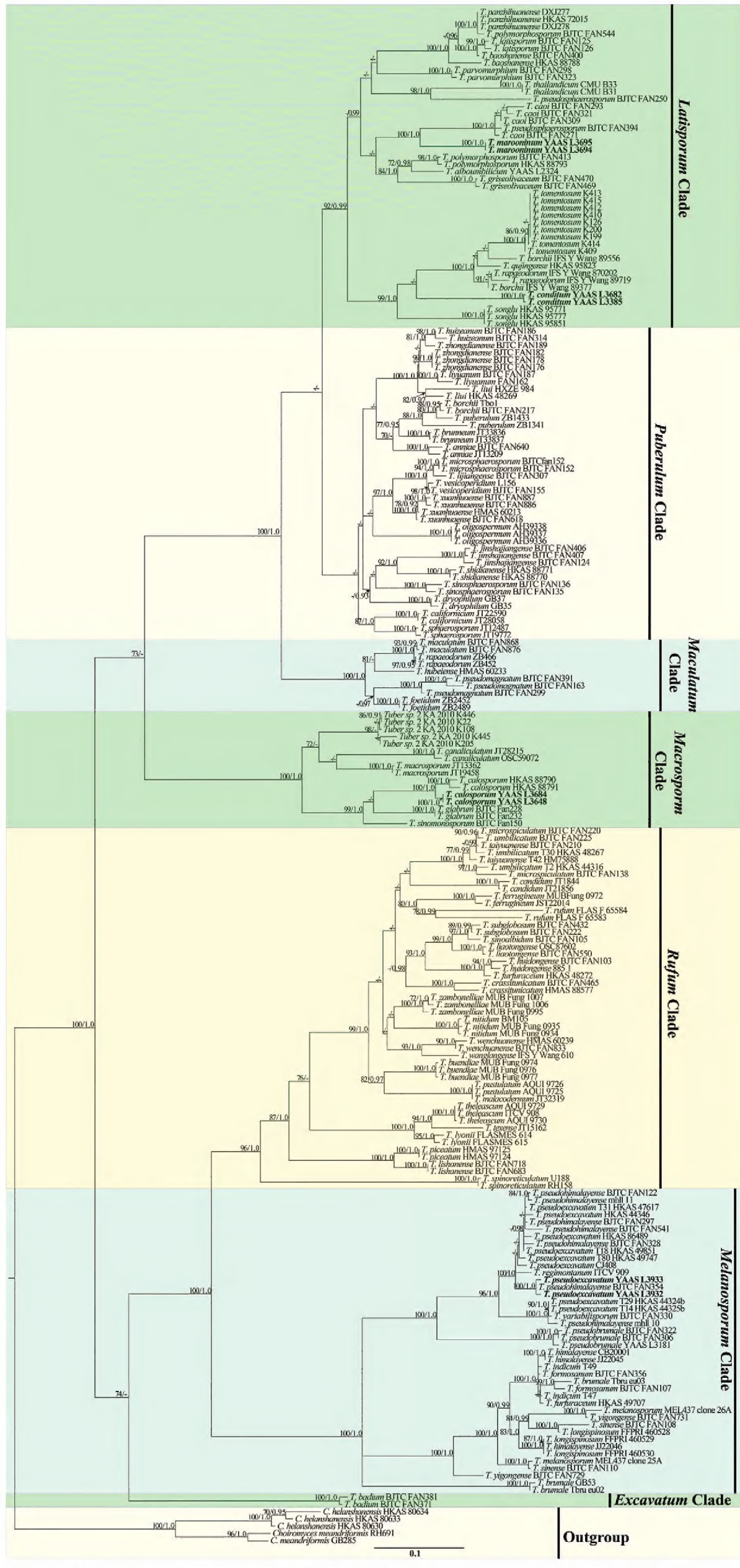

Figure 2. Phylogeny generated from maximum likelihood and Bayesian inference analysis of the ITS1-5.8S-ITS2 sequences from *Tuber marroninum* and *T. conditum* related species. *Choiromyces helanshanensis* and *C. meandriformis* served as outgroups. ML bootstrap values (>75%) and Posterior Probabilities (PPs) values (>0.90) are shown above or beneath the branches at nodes. Novel sequences are printed in bold.

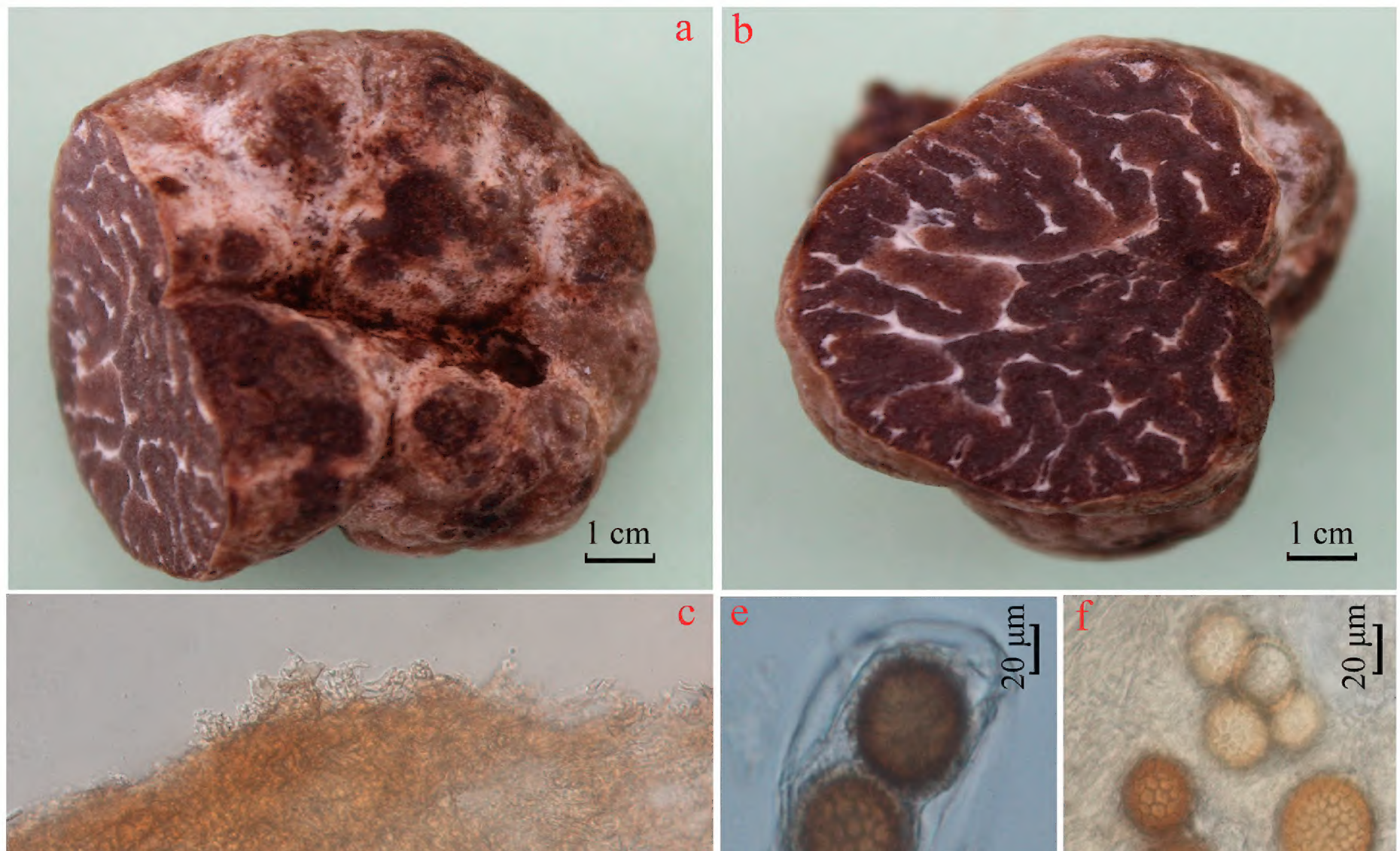

Figure 3. Photographs of *Tuber marroninum* (YAAS L3694, holotype): **a** fresh ascomata showing cut section **b** the gleba **c, d** vertical section of peridium **d** the gleba and asci **e, f** ascospores **g** scanning electron micrograph of ascospore.

Gene sequences ex holotype. ON454668 (ITS); ON428904 (LSU); OQ305202 (RPB2); OQ305199 (TEF1); ex paratype: OQ297680 (ITS).

Etymology. "Marroninum" refers to the maroon color of the ascoma.

Diagnosis. *Tuber marroninum* differs from other species by its whitish and glabrous ascomata and maroon color of the ascoma, globose and subglobose or broad ellipsoid ascospores, with greyish-white alveolate-reticulate ornamentation.

Description. ***Ascomata*** (Fig. 3a, b) 3–5 cm diam, subglobose, hypogeous, whitish (3A1-2) becoming brown (8E6-8) when bruised, surface smooth, with white furrows; odor mild, taste not recorded. ***Peridium*** (Fig. 3c, d) two-layered, 150–200 µm thick, outer layer 40–60 µm thick, pseudoparenchymatous, composed of subglobose or irregularly shaped cells of 7.5–16.8 (24.5) µm broad with thickened walls, inner layer composed of thin-walled hyaline interwoven hyphae, 2.5–4 (5.5) µm diam, large cells of 20 × 10 µm diam. Sometimes, intermixed. ***Gleba*** (Fig. 3b) solid, firm, brown to dark brown (10F6-8) at maturity, marbled with whitish (1A1-1B1) narrow veins. ***Asci*** (Fig. 3e, g) subglobose or irregular, 1–4 spored, with a short stalk, 50–75 (100) × 40–55 (80) µm (n = 50). ***Ascospores*** (Fig. 3e, g) subglobose to globose, pale yellow (5A2-3, 5B3) when young, becoming brown (5C6-8, 5D6-7) at maturity, excluding their spiny-reticulate ornamentation, 41.5–52 × 41–50 µm, *Q* = 1.00–1.14 (n = 55), in one-spored asci, 33–41 × 32–40 µm (n = 50), *Q* = 1.00–1.15, in two-spored asci, 21–37 × 20–35.5 µm, *Q* = 1.01–1.12 (n = 60), in three-spored asci, 20–35.5 × 18.5–30.5 µm, *Q* = 1.02–1.15 (n = 60), in four-spored asci, *Q* = 1.14 ± 0.07, with a greyish white (2B1-2; 1C1-2) reticulatum and alveolate-reticulate ornamentation, meshes 5–8 across the ascospore width, 1.5–3 µm tall.

Distribution and habitat. China: Yunnan province, Xiangyun county, hypogeous, in the soil in mixed woods dominated by *Pinus yunnanensis*.

Notes. *Tuber marroninum* is phylogenetically closely related to *T. caoi* (Fan et al. 2016). In morphology, *T. caoi* has gray and pubescent ascomata, but *T. marroninum* has whitish and glabrous ascomata. Both trees of ML and BI (Figs 1, 2) also have strong bootstrap supporting the new species.

Tuber conditum **T. J. Yuan, S. H. Li & X. H. Wang, sp. nov.**

MycoBank No: 901882

Fig. 4a–f

Typification. China • Yunnan Province: Changning County (102.3535°E, 38.3903°E), in soil under mixed forest, with *Pinus yunnanensis* dominant, 20 Sep 2020, S. H. Li L3385 (holotype YAAS L3385, paratype YAAS L3682).

Gene sequences ex holotype. ON454665 (ITS); ON428901 (LSU); ex paratype: ON454667 (ITS).

Etymology. Conditum, Latin, refers to the acrid smell of ascocarp.

Diagnosis. *Tuber conditum* differs from other species by having no cystidia on surface of ascomata, thinner peridium, and larger ellipsoid ascospores.

Description. ***Ascomata*** (Fig. 4a) 3–4 cm diam., subglobose, hypogeous, grey-white to grey-brown (4A2-4; 4B3-4), surface smooth, with a few pinholes; acrid smell, taste not recorded. ***Peridium*** (Fig. 4b) one-layered, 120–200 µm thick, prosenchymatous, composed of interwoven hyphae 1.5–2.5 µm broad with thin-walled, and lumens 1.5–2.5 (3.5) µm diam, with ellipsoid or irregular cells of 15 × 10 µm diam. Sometimes intermixed, wall thickness 1–2 µm. ***Gleba*** (Fig. 4a, c) solid, firm, brown to black (6E7-8; (5-9)F8) at maturity, marbled with whitish narrow veins. ***Asci*** (Fig. 4d–e) subglobose or irregular, 1–4 spored, hyaline, thin-walled or occasionally with walls as thick as 2 µm, sessile stalk, 55–100 × 45–75 µm (n = 30). ***Ascospores*** (Fig. 4d–f) subglobose or ellipsoid, pale yellow (2A2-4), yellow-brown (5D6-8; 5E7-8) at maturity, excluding orna-

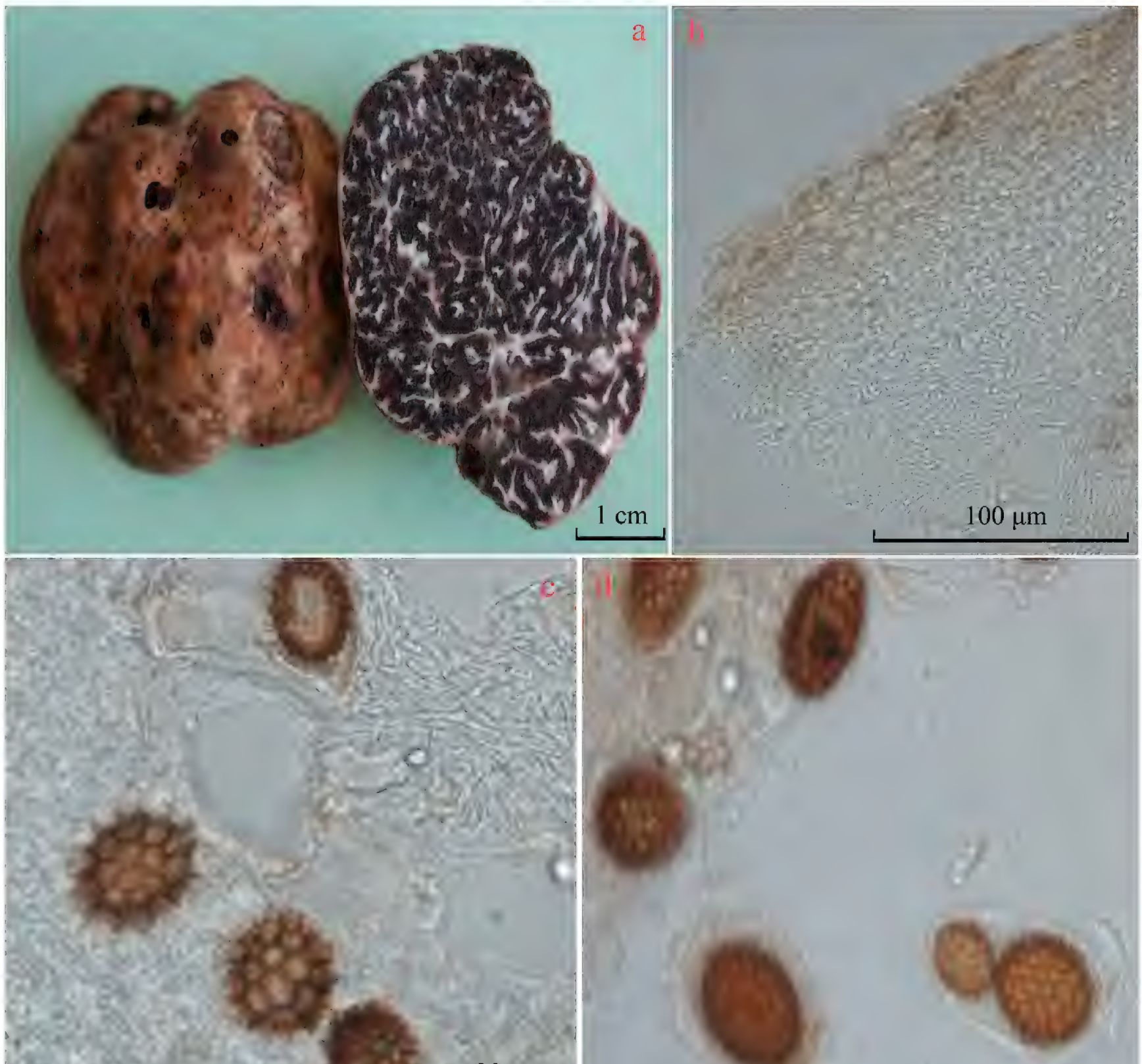

Figure 4. Photographs of *Tuber conditum* (YAAS L3385, holotype): **a** fresh ascomata showing cut section **b** vertical section of peridium **c, d** the gleba and asci **e** ascospores **f** scanning electron micrograph of ascospore.

mentation, 49–66.5 × 34.5–60 µm, *Q* = 1.02–1.58 (n = 54), in one-spored asci, 33–54.5 × 30–47 µm, *Q* = 1.06–1.35 (n = 50), in two-spored asci, 21.5–44 × 21–37 µm, *Q* = 1.00–1.26 (n = 60), in three-spored asci, 20.5–40.0 × 20–35.5 µm, *Q* = 1.01–1.30 (n = 40), in four-spored asci, Q = 1.15 ± 0.23, reticulate ornamentation 2–5 µm in height, composed of irregular hexagonal meshes, 6–8 along the spore length and 3–6 along the breadth.

Distribution and habitat. China: Yunnan province, Changning county, hypogeous, in the soil in woods dominated by *Pinus yunnanensis*.

Notes. *Tuber conditum* is phylogenetically closely related to *Tuber tomentosum* (Kinoshita et al. 2011), *T. qujingense*, and *T. songlu* (Wan et al. 2021). However, *T. conditum* has no cystidia on the surface of ascomata and larger ellipsoid ascospores, differing from *T. tomentosum* (Kinoshita et al. 2011; Sasaki et al. 2016). *Tuber conditum* differs from *T. qujingense* and *T. songlu* by its thinner peridium (120–200 µm) and larger ascospores (49–66.5 × 34.5–60 µm in one-spored asci). *Tuber qujingense* has a greyish-white ascomata, brown snowflake-shaped gleba, and prosenchymatous peridium, fusiform, and *T. songlu* has dense spine-like dermatocystidia, which are completely different from each other. In addition, DNA sequence analysis revealed the similarity between two species is less than 82.73% (*T. qujingense*) and 93% (*T. songlu*) in ITS sequences, strongly supporting the separation of *T. conditum* from the Chinese species of *T. qujingense* and *T. songlu.* Phylogenetic analysis (Figs 1, 2) also suggested that *T. conditum* is a distinct species because all sequences of this new species clustered within a strong bootstrap (BS ≥ 99% and PP = 1.0). The truffle has the acrid smell, which might be a good identifying feature for the fungus.

***Tuber pseudoexcavatum* Y. Wang et al., Cryptogamie Mycologie, 1998, 19(1–2): 113–120 (Manjón et al. 2009; Moreno et al. 1997)**

Notes. The description of *Tuber pseudoexcavatum* Y. Wang et al. (1998) was originally based on two specimens collected in China and published by Wang et al. in 1998. Subsequently, additional collections (AH 18387, AH 18331) from China were successfully used to generate DNA data, which were deposited in the Herbarium of the Institute of Forestry and Soil Sciences. This study reports two new collections that suggest the presence of this species may not be uncommon in the southwestern region of China. Morphologically, *T. pseudoexcavatum* is characterized by subglobose ascocarps with a deeply excavate appearance, ranging in color from brown to brown-orange. The surface exhibits a coarsely warted texture, while the asci contain 1–8 ascospores with spinoreticulate ornamentation, which may serve as diagnostic features based on the current collections. The known distribution of this species is limited to China.

Material examined. Location • Yunnan Province, Changning County (37.1212°N,). Habitat: Hypogeous, found in soil beneath a pure *Pinus armandii* forest Altitude: Approximately 2500 m Collection Date: 20 Sep. 2020 Specimens: YAAS L3932, YAAS L3933 Collector: S. H. Li Repository: Herbarium of Institute of Forestry and Soil Sciences (YAAS).

***Tuber calosporum* S. P. Wan et al. Mycoscience, 2016, 57(6): 393–399**

Note. *Tuber calosporum* S. P. Wan et al. was originally described based on four specimens collected from Huize County in Yunnan Province, as detailed by Wan et al. in 2016. The specimens (wsp352, wsp145, wsp186, and wsp382) provided successful DNA data. This study introduces two additional collections, suggesting a broader distribution of the species in the southwest region of China. Morphologically, *T. calosporum* is recognized by its brownish-yellow ascocarps with a verrucose surface, featuring superficial yellow furrows. The ascospores are characterized by their large, ellipsoid shape and shallow alveolar ornamentation, which can serve as diagnostic traits based on the present collections. The species is known to occur exclusively in China.

Material examined. Location • Yunnan Province, Xiangyun County (25°39'43"N, 100°52'27"E) Habitat: Found in soil beneath a mixed forest dominated by Pinus yunnanensis Collection Date: 20 Sep 2020 Specimens: YAAS L3648, YAAS L3684 Collector: S. H. Li Repository: Herbarium of Institute of Forestry and Soil Sciences (YAAS).

Discussion

The similarities of ITS sequences of *T. marroninum* and *T. conditum* to all described *Tuber* species are lower than 95.5% and 89.0%, respectively. In the genus *Tuber*, species delimitation based on 96% similarity in ITS has been suggested by previous studies (Kinoshita et al. 2011, 2021). Moreover, *T. marroninum* and *T. conditum* can be distinguished from other related species by morphology alone. In addition, phylogenetically, *T. marroninum* forms a distinct branch with high support (ITS 100% BS and 1.0 PP), and its sister species, *T. caoi*, can be distinguished by ITS-LUS-TEF1-RPB2 and ITS1 + 5.8S + ITS2 combined ML trees (Figs 1, 2). *Tuber conditum* can be distinguished from *T. tomentosum*, *T. rapaeodorum*, and *T. songlu* by the ML tree of ITS-LUS-TEF1-RPB2 (Fig. 1) and from *T. tomentosum* by the ITS1 + 5.8S + ITS2 combined ML tree (Fig. 2).

Morphologically, *T. marroninum* resembles *T. caoi*, *T. jinshajiangense* (Fan et al. 2016), and *T. shii* (Wang et al. 2016) of Chinese species with regard to its regularly globose ascospores with alveolar ornamentation. However, *T. caoi* and *T. jinshajiangense* have gray or pale gray and pubescent ascomata, but *T. marroninum* has whitish and glabrous ascomata. There are obvious differences between both *T. marroninum* and *T. shii* from their ascomata and gleba. *Tuber shii* often has some superficial furrows, is pale grey-brown or pale brown, and has distinctive silver-grey tints ascomata, its gleba with brown at maturity (Wang et al. 2016), but *T. marroninum* has whitish and brown glabrous ascomata, and its gleba is brown to dark brown at maturity. Additionally, *T. marroninum* differs from *T. caoi* (37.5–40 µm in one-spored asci), *T. jinshajiangense* (35–37.5 µm in one-spored asci), and *T. shii* (29–51.5 µm in one-spored asci) by its larger ascospore (41.5–52 µm in one-spored asci). *Tuber conditum* differs from other species by its grey-white to grey-brown ascomata, thinner peridium (120–200 µm), and larger ascospores (49–66.5 × 34.5–60 µm in one-spored asci). The close relationship species of *T. tomentosum* has abundant spiny cystidia on the peridium surface (Kinoshita et al. 2011), and *T. pseudosphaerosporum*

has 2–4.5 cm diam and white or whitish-yellow ascomata (Fan and Yue 2013). Furthermore, *T. marroninum* and *T. conditum* can remarkably identify other Chinese species of the Latisporum group, such as *T. alboumbilicum* (Li et al. 2014), *T. baoshanense* (Wan et al. 2017), *T. elevatireticulatum* (Lin et al. 2018), *T. griseolivaceum* (Huang et al. 2017), *T. huiliense*, *T. luyashanense*, *T. microcarpum* (Fan et al. 2022), and *T. panzhihuanense* (Deng et al. 2013), based on the color and size of ascomata. Of course, the two pairs of truffles are quite different because the phylogenetic tree places them in a different position by ITS-LUS-TEF1-RPB2 and ITS1 + 5.8S + ITS2 combined ML trees (Figs 1, 2).

In conclusion, we demonstrated that *T. marroninum* is a new species characterized by the maroon color of the ascoma, and *T. conditum* is a new species characterized by its smooth ascocarp surfaces and larger ellipsoid ascospores. Our findings of these species, *T. conditum*, *T. songlu*, *T. qujingense*, *T. rapaeodorum*, and *T. tomentosum*, are divided within the same group by their morphologic characteristics and phylogenetic relationships. In addition, *T. marroninum*, *T. pseudosphaerosporum*, and *T. caoi* are divided within the same group by their morphologic characteristics and phylogenetic relationships (Figs 1, 2). However, they were revealed to possibly belong to the Latisporum group (Wan et al. 2016, 2017; Fan et al. 2022) by the phylogenetic relationships through adding more DNA sequences in this study.

Key to the species of the Latisporum group

1 One-layer peridium **2**
– Two-layer peridium **4**
2 Peridium prosenchymatous **3**
– Peridium pseudoparenchymatous **5**
3 Irregular and lobed ..... ***T. panzhihuanense* (Deng et al., 2013)**
– Surface smooth and with a few pinholes ..... ***T. conditum***
4 Peridium smooth and glabrous **6**
– Peridium puberulent **7**
5 Ascomata white umbilicate ..... ***T. alboumbilicum* (Li et al. 2014)**
– Ascomata whitish, becoming brown when bruised with white furrow ..... ***T. marroninum***
6 Ascomata whitish-yellow and mostly much lobed with deep furrows ..... ***T. pseudosphaerosporum* (Fan & Yue, 2013)**
– Ascomata pale yellow to desert yellow with distinctively hygrophanous patch around or near the base ..... ***T. parvomurphium* (Fan et al., 2016)**
7 Ascospores mainly globose **8**
– Ascospores mainly broadly ellipsoid **9**
8 Ascospores globose only ..... ***T. caoi* (Fan et al., 2016)**
– Ascospores fusiform, globose or sometimes subglobose ..... ***T. polymorphosporum* (Wan et al., 2017)**
9 Ascomata olive gray or gray with green ..... ***T. griseolivaceum* (Huang et al., 2017)**
– Ascomata pale yellowish brown or reddish brown **10**
10 Ascospores broad ellipsoid **11**
– Ascospores ellipsoid, subglobose **12**

11 Asci 2–3 spored, ascospores smaller (25–40 × 14–25 µm)..... ..... ***Tuber tomentosum*** **(Kinoshita et al., 2011)**

– Asci 1–4 spored, ascospores larger (30–60 × 25–50 µm) .... ***Tuber*** **sp. 12 (Kinoshita et al., 2011)**

12 Ascomata ocher-yellow **13**

– Ascomata whitish.....**14**

13 Asci 2–3 spored, ascospores smaller (30–35 × 20–30 µm)..... ..... ***Tuber*** **sp. 11 (Kinoshita et al., 2011)**

– Asci 2–3 spored, ascospores largerer (30–55 × 30–40 µm) .... ***Tuber*** **sp. 13 (Kinoshita et al., 2011)**

14 Ascospores reddish-brown, with five-spored in asci and alveolar walls up to 2–6 µm tall .....***T. baoshanense*** **(Wan et al., 2017)**

– Ascospores yellowish brown to brown, with four-spored in asci and alveolate reticulum 3–5 µm tall.....***T. thailandicum*** **(Suwannarach et al., 2015)**

– Ascospores yellowish brown to reddish brown, with four-spored in asci and alveolar walls up to 3–5 µm deep.... ***T. latisporum*** **(Chen & Liu, 2007)**

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

All authors have contributed equally.

Data availability

All of the data that support the findings of this study are available in the main text.

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